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APPLICATION

FOR

UNITED STATES LETTERS PATENT

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TITLE : *RPS* GENE FAMILY, PRIMERS, PROBES, AND DETECTION METHOD

RPS GENE FAMILY, PRIMERS, PROBES, AND DETECTION METHODS

Statement as to Federally Sponsored Research

This invention was made in part with Government funding and the Government therefore has certain rights in the invention.

Background of the Invention

This application is a continuation-in-part of application serial number 08/227,360, filed April 13, 1994.

The invention relates to recombinant plant nucleic acids and polypeptides and uses thereof to confer disease resistance to pathogens in transgenic plants.

Plants employ a variety of defensive strategies to combat pathogens. One defense response, the so-called hypersensitive response (HR), involves rapid localized necrosis of infected tissue. In several host-pathogen interactions, genetic analysis has revealed a gene-for-gene correspondence between a particular avirulence (*avr*) gene in an avirulent pathogen that elicits an HR in a host possessing a particular resistance gene.

Summary of the Invention

In general, the invention features substantially pure DNA (for example, genomic DNA, cDNA, or synthetic DNA) encoding an Rps polypeptide as defined below. In related aspects, the invention also features a vector, a cell (e.g., a plant cell), and a transgenic plant or seed thereof which includes such a substantially pure DNA encoding an Rps polypeptide.

In preferred embodiments, an *RPS* gene is the *RPS2* gene of a plant of the genus *Arabidopsis*. In various

preferred embodiments, the cell is a transformed plant cell derived from a cell of a transgenic plant. In related aspects, the invention features a transgenic plant containing a transgene which encodes an Rps polypeptide that is expressed in plant tissue susceptible to infection by pathogens expressing the *avrRpt2* avirulence gene or pathogens expressing an avirulence signal similarly recognized by an Rps polypeptide.

In a second aspect, the invention features a substantially pure DNA which includes a promoter capable of expressing the *RPS2* gene in plant tissue susceptible to infection by bacterial pathogens expressing the *avrRpt2* avirulence gene.

In preferred embodiments, the promoter is the promoter native to an *RPS* gene. Additionally, transcriptional and translational regulatory regions are preferably native to an *RPS* gene.

The transgenic plants of the invention are preferably plants which are susceptible to infection by a pathogen expressing an avirulence gene, preferably the *avrRpt2* avirulence gene. In preferred embodiments the transgenic plant is from the group of plants consisting of but not limited to *Arabidopsis*, tomato, soybean, bean, maize, wheat and rice.

In another aspect, the invention features a method of providing resistance in a plant to a pathogen which involves: (a) producing a transgenic plant cell having a transgene encoding an Rps2 polypeptide wherein the transgene is integrated into the genome of the transgenic plant and is positioned for expression in the plant cell; and (b) growing a transgenic plant from the transgenic plant cell wherein the *RPS2* transgene is expressed in the transgenic plant.

In another aspect, the invention features a method of detecting a resistance gene in a plant cell involving: (a) contacting the *RPS2* gene or a portion thereof greater than 9 nucleic acids, preferably greater than 18 nucleic acids in length with a preparation of genomic DNA from the plant cell under hybridization conditions providing detection of DNA sequences having about 50% or greater sequence identity to the DNA sequence of Fig. 2 encoding the Rps2 polypeptide.

In another aspect, the invention features a method of producing an Rps2 polypeptide which involves: (a) providing a cell transformed with DNA encoding an Rps2 polypeptide positioned for expression in the cell; (b) culturing the transformed cell under conditions for expressing the DNA; and (c) isolating the Rps2 polypeptide.

In another aspect, the invention features substantially pure Rps2 polypeptide. Preferably, the polypeptide includes a greater than 50 amino acid sequence substantially identical to a greater than 50 amino acid sequence shown in Fig. 2, open reading frame "a". Most preferably, the polypeptide is the *Arabidopsis thaliana* Rps2 polypeptide.

In another aspect, the invention features a method of providing resistance in a transgenic plant to infection by pathogens which do not carry the *avrRpt2* avirulence gene wherein the method includes: (a) producing a transgenic plant cell having transgenes encoding an Rps2 polypeptide as well as a transgene encoding the *avrRpt2* gene product wherein the transgenes are integrated into the genome of the transgenic plant; are positioned for expression in the plant cell; and the *avrRpt2* transgene and, if desired, the *RPS2* gene, are under the control of regulatory sequences suitable for controlled expression of the gene(s); and (b) growing a

transgenic plant from the transgenic plant cell wherein the *RPS2* and *avrRpt2* transgenes are expressed in the transgenic plant.

In another aspect, the invention features a method of providing resistance in a transgenic plant to infection by pathogens in the absence of avirulence gene expression in the pathogen wherein the method involves: (a) producing a transgenic plant cell having integrated in the genome a transgene containing the *RPS2* gene under the control of a promoter providing constitutive expression of the *RPS2* gene; and (b) growing a transgenic plant from the transgenic plant cell wherein the *RPS2* transgene is expressed constitutively in the transgenic plant.

In another aspect, the invention features a method of providing controllable resistance in a transgenic plant to infection by pathogens in the absence of avirulence gene expression in the pathogen wherein the method involves: (a) producing a transgenic plant cell having integrated in the genome a transgene containing the *RPS2* gene under the control of a promoter providing controllable expression of the *RPS2* gene; and (b) growing a transgenic plant from the transgenic plant cell wherein the *RPS2* transgene is controllably expressed in the transgenic plant. In preferred embodiments, the *RPS2* gene is expressed using a tissue-specific or cell type-specific promoter, or by a promoter that is activated by the introduction of an external signal or agent, such as a chemical signal or agent.

In other aspects, the invention features a substantially pure oligonucleotide including one or a combination of the sequences:

5' GGNATGGGNGGNNTNGGNAARACNAC 3', wherein N is A, T, G, or C; and R is A or G;

5' NARNGGNARNCC 3', wherein N is A, T, G or C; and R is A or G;

5'NCGNGWNGTNAKDAWNCGNGA 3', wherein N is A, T, G or C; W is A or T; D is A, G, or T; and K is G or T;

5' GGWNTBGGWAARACHAC 3', wherein N is A, T, G or C; R is G or A; B is C, G, or T; H is A, C, or T; and W is A or T;

5' TYGAYGAYRTBKRBRA 3', wherein R is G or A; B is C, G, or T; D is A, G, or T; Y is T or C; and K is G or T;

5' TYCCAVAYRTCRTCNA 3', wherein N is A, T, G or C; R is G or A; V is G or C or A; and Y is T or C;

5' GGWYTBCCWYTBGCHYT 3', wherein B is C, G, or T; H is A, C, or T; W is A or T; and Y is T or C;

5' ARDGCVARWGGVARNCC 3', wherein N is A, T, G or C; R is G or A; W is A or T; D is A, G, or T; and V is G, C, or A; and

5' ARRTTRTCRTADSWRAWYTT 3', wherein R is G or A; W is A or T; D is A, G, or T; S is G or C; and Y is C or T.

In other aspects, the invention features a recombinant plant gene including one or a combination of the DNA sequences:

5' GGNATGGGNGGNNTNGGNAARACNAC 3', wherein N is A, T, G or C; and R is A or G;

5' NARNGGNARNCC 3', wherein N is A, T, G or C; and R is A or G;

5' NCGNGWNGTNAKDAWNCGNGA 3', wherein N is A, T, G or C; W is A or T; D is A, G or T; and K is G or T.

In another aspect, the invention feaures a substantially pure plant polypeptide including one or a combiantion of the amino acid sequences:

Gly Xaa_1 Xaa_2 Gly Xaa_3 Gly Lys Thr Thr Xaa_4 Xaa_5, wherein Xaa_1 is Met or Pro; Xaa_2 is Gly or Pro; Xaa_3 is Ile,

Leu, or Val; Xaa_4 is Ile, Leu, or Thr; and Xaa_5 is Ala or Met;

Xaa_1 Xaa_2 Xaa_3 Leu Xaa_4 Xaa_5 Xaa_6 Asp Asp Xaa_7 Xaa_8, wherein Xaa_1 is Phe or Lys; Xaa_2 is Arg or Lys; Xaa_3 is Ile, Val, or Phe; Xaa_4 is Ile, Leu, or Val; Xaa_5 is Ile or Leu; Xaa_6 is Ile or Val; Xaa_7 is Ile, Leu, or Val; and Xaa_8 is Asp or Trp;

Xaa_1 Xaa_2 Xaa_3 Xaa_4 Xaa_5 Thr Xaa_6 Arg, wherein Xaa_1 is Ser or Cys; Xaa_2 is Arg or Lys; Xaa_3 is Phe, Ile, or Val; Xaa_4 is Ile, or Met; Xaa_5 is Ile, Leu, or Phe; Xaa_6 is Ser, Cys, or Thr;

Gly Leu Pro Leu Xaa_1 Xaa_2 Xaa_3 Xaa_4, wherein Xaa_1 is Thr, Ala, or Ser; Xaa_2 is Leu or Val; Xaa_3 is Ile, Val, or Lys; and Xaa_4 is Val or Thr; and

Xaa_1 Xaa_2 Ser Tyr Xaa_3 Xaa_4 Leu, wherein Xaa_1 is Lys or Gly; Xaa_2 is Ile or Phe; Xaa_3 is Asp or Lys; and Xaa_4 is Ala, Gly, or Asn.

In another aspect, the invention features a method of isolating a disease-resistance gene or fragment thereof from a plant cell, involving: (a) providing a sample of plant cell DNA; (b) providing a pair of oligonucleotides having sequence homology to a conserved region of an RPS disease-resistance gene; (c) combining the pair of oligonucleotides with the plant cell DNA sample under conditions suitable for polymerase chain reaction-mediated DNA amplification; and (d) isolating the amplified disease-resistance gene or fragment thereof.

In preferred embodiments, the amplification is carried out using a reverse-transcription polymerase chain reaction, for example, the RACE method

In another aspect, the invention features a method of identifying a plant disease-resistance gene in a plant cell, involving: (a) providing a preparation of plant cell DNA (for example, from the plant genome); (b) providing a detectably-labelled DNA sequence (for example, prepared by the methods of the invention) having homology to a conserved region of an RPS gene; (c) contacting the preparation of plant cell DNA with the detectablly-labelled DNA sequence under hybridization conditions providing detection of genes having 50% or greater sequence identity; and (d) identifying a disease-resistance gene by its association with the detectable label.

In another aspect, the invention features a method of isolating a disease-resistance gene from a recombinant plant cell library, involving: (a) providing a recombinant plant cell library; (b) contacting the recombinant plant cell library with a detectably-labelled gene fragment produced according to the PCR method of the invention under hybridization conditions providing detection of genes having 50% or greater sequence identity; and (c) isolating a member of a disease-resistance gene by its association with the detectable label.

In anotehr aspect, the invention features a method of isolating a disease-resistance gene from a recombinant plant cell library, involving: (a) providing a recombinant plant cell library; (b) contacting the recombinant plant cell library with a detectably-labelled RPS oligonucleotide of the invention under hybridization conditions providing detection of genes having 50% or greater sequence identity;

and (c) isolating a disease-resistance gene by its association with the detectable label.

In another aspect, the invention features a recombinant plant polypeptide capable of conferring disease-resistance wherein the plant polypeptide includes a P-loop domain or nucleotide binding site domain. Preferably, the polypeptide further includes a leucine-rich repeating domain.

In another aspect, the invention features a recombinant plant polypeptide capable of conferring disease-resistance wherein the plant polypeptide contains a leucine-rich repeating domain.

In anotehr aspect, the invention features a plant disease-resistance gene isolated according to the method involving: (a) providing a sample of plant cell DNA; (b) providing a pair of oligonucleotides having sequence homology to a conserved region of an RPS disease-resistance gene; (c) combining the pair of oligonucleotides with the plant cell DNA sample under conditions suitable for polymerase chain reaction-mediated DNA amplification; and (d) isolating the amplified disease-resistance gene or fragment thereof.

In another aspect, the invention features a plant disease-resistance gene isolated according to the method involving: (a) providing a preparation of plant cell DNA; (b) providing a detectably-labelled DNA sequence having homology to a conserved region of an RPS gene; (c) contacting the preparation of plant cell DNA with the detectably-labelled DNA sequence under hybridization conditions providing detection of genes having 50% or greater sequence identity; and (d) identifying a disease-resistance gene by its association with the detectable label.

In another aspect, the invention features a plant disease-resistance gene according to the method involving: (a) providing a recombinant plant cell library; (b) contacting the recombinant plant cell library with a detectably-labelled RPS gene fragment produced according to the method of the invention under hybridization conditions providing detection of genes having 50% or greater sequence identity; and (c) isolating a disease-resistance gene by its association with the detectable label.

In another aspect, the invention features a method of identifying a plant disease-resistance gene involving: (a) providing a plant tissue sample; (b) introducing by biolistic transformation into the plant tissue sample a candidate plant disease-resistance gene; (c) expressing the candidate plant disease-resistance gene within the plant tissue sample; and (d) determining whether the plant tissue sample exhibits a disease-resistance response, whereby a response identifies a plant disease-resistance gene.

Preferably, the plant tissue sample is either leaf, root, flower, fruit, or stem tissue; the candidate plant disease-resistance gene is obtained from a cDNA expression library; and the disease-resistance response is the hypersensitive response.

In another aspect, the invention features a plant disease-resistance gene isolated according to the method involving: (a) providing a plant tissue sample; (b) introducing by biolistic transformation into the plant tissue sample a candidate plant disease-resistance gene; (c) expressing the candidate plant disease-resistance gene within the plant tissue sample; and (d) determining whether the plant tissue sample exhibits a disease-resistance response, whereby a response identifies a plant disease-resistance gene.

In another aspect, the invention features a purified antibody which binds specifically to an rps family protein. Such an antibody may be used in any standard immunodetection method for the identification of an RPS polypeptide.

In another aspect, the invention features a DNA sequence substantially identical to the DNA sequence shown in Figure 12.

In another aspect, the invention features a substantially pure polypeptide having a sequence substantially identical to a Prf amino acid sequence shown in Figure 5 (A or B).

By "disease resistance gene" is meant a gene encoding a polypeptide capable of triggering the plant defense response in a plant cell or plant tissue. An *RPS* gene is a disease resistance gene having about 50% or greater sequence identity to the *RPS2* sequence of Fig. 2 or a portion thereof. The gene, *RPS2*, is a disease resistance gene encoding the Rps2 disease resistance polypeptide from *Arabidopsis thaliana*.

By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%, preferably 85%, more preferably 90%, and most preferably 95% homology to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more

preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Sequence identity is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By a "substantially pure polypeptide" is meant an Rps2 polypeptide which has been separated from components which naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, Rps2 polypeptide. A substantially pure Rps2 polypeptide may be obtained, for example, by extraction from a natural source (e.g., a plant cell); by expression of a recombinant nucleic acid encoding an Rps2 polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., those described in column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus,

a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) an Rps2 polypeptide.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., an Rps2 polypeptide, a recombinant protein or a RNA molecule).

By "reporter gene" is meant a gene whose expression may be assayed; such genes include, without limitation, β-glucuronidase (GUS), luciferase, chloramphenicol transacetylase (CAT), and ß-galactosidase.

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention are

those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5′ or 3′ regions of the native gene.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

By "plant cell" is meant any self-propagating cell bounded by a semi-permeable membrane and containing a plastid. Such a cell also requires a cell wall if further propagation is desired. Plant cell, as used herein includes, without limitation, algae, cyanobacteria, seeds suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell which includes a DNA sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic plants and the DNA (transgene) is inserted by artifice into the nuclear or plastidic genome.

By "pathogen" is meant an organism whose infection into the cells of viable plant tissue elicits a disease response in the plant tissue.

By an "RPS disease-resistance gene" is meant any member of the family of plant genes characterized by their ability to trigger a plant defense response and having at least 20%, preferably 30%, and most preferably 50% amino acid sequence identity to one of the conserved regions of one of the RPS members described herein (i.e., either the RPS2, L6, N, or Prf genes). Representative members of the RPS gene family include, without limitation, the rps2 gene of *Arabidopsis*, the L6 gene of flax, the Prf gene of tomato, and the N gene of tobacco.

By "conserved region" is meant any stretch of six or more contiguous amino acids exhibiting at least 30%, preferably 50%, and most preferably 70% amino acid sequence identity between two or more of the RPS family members, RPS2, L6, N, or Prf. Examples of preferred conserved regions are shown (as boxed or designated sequences) in Figures 5 A and B, 6, 7, and 8 and include, without limitation, nucleotide binding site domains, leucine-rich repeats, leucine zipper domains, and P-loop domains.

By "detectably-labelled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule. Methods for detectably-labelling a molecule are well known in the art and include, without limitation, radioactive labelling (e.g., with an isotope such as ^{32}P or ^{35}S) and nonradioactive labelling (e.g., chemiluminescent labelling, e.g., fluorescein labelling).

By "biolistic transformation" is meant any method for introducing foreign molecules into a cell using velocity

driven microprojectiles such as tungsten or gold particles. Such velocity-driven methods originate from pressure bursts which include, but are not limited to, helium-driven, air-driven, and gunpowder-driven techniques. Biolistic transformation may be applied to the transformation or transfection of a wide variety of cell types and intact tissues including, without limitation, intracellular organelles (e.g., chloroplasts and mitochondria), bacteria, yeast, fungi, algae, pollen, animal tissue, plant tissue (e.g., leaf, seedling, embryo, epidermis, flower, meristem, and root), pollen, and cultured cells.

By "purified antibody" is meant antibody which is at least 60%, by weight, free from proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably 90%, and most preferably at least 99%, by weight, antibody, e.g., an rps2-specific antibody. A purified rps antibody may be obtained, for example, by affinity chromatography using recombinantly-produced rps protein or conserved motif peptides and standard techniques.

By "specifically binds" is meant an antibody which recognizes and binds an rps protein but which does not substantially recognize and bind other molecules in a sample, e.g., a biological sample, which naturally includes rps protein.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

The drawings will first be described.

Drawings

Figs. 1A - 1F are a schematic summary of the physical and RFLP analysis that led to the cloning of the *RPS2* locus.

Fig. 1A is a diagram showing the alignment of the genetic and the RFLP maps of the relevant portion of *Arabidopsis thaliana* chromosome IV adapted from the map published by Lister and Dean (1993) Plant J. 4:745-750. The RFLP marker L11F11 represents the left arm of the YUP11F11 YAC clone.

Fig. 1B is a diagram showing the alignment of relevant YACs around the *RPS2* locus. YAC constructs designated YUP16G5, YUP18G9 and YUP11F11 were provided by J. Ecker, University of Pennsylvania. YAC constructs designated EW3H7, EW11D4, EW11E4, and EW9C3 were provided by E. Ward, Ciba-Geigy, Inc.

Fig. 1C is a diagram showing the alignment of cosmid clones around the *RPS2* locus. Cosmid clones with the designation H are derivatives of the EW3H7 YAC clone whereas those with the designation E are derivatives of the EW11E4 YAC clone. Vertical arrows indicate the relative positions of RFLP markers between the ecotypes La-er and the *rps2-101N* plant. The RFLP markers were identified by screening a Southern blot containing more than 50 different restriction enzyme digests using either the entire part or pieces of the corresponding cosmid clones as probes. The cosmid clones described in Fig. 1C were provided by J. Giraudat, C.N.R.S., Gif-sur-Yvette, France.

Figs. 1D and 1E are maps of *Eco*RI restriction endonuclease sites in the cosmids E4-4 and E4-6, respectively. The recombination break points surrounding the *RPS2* locus are located within the 4.5 and 7.5 kb *Eco*RI restriction endonuclease fragments.

Fig. 1F is a diagram showing the approximate location of genes which encode the RNA transcripts which have been identified by polyA$^+$ RNA blot analysis. The sizes of the transcripts are given in kilobase pairs below each transcript.

Fig. 2 is the complete nucleotide sequence of cDNA-4 comprising the *RPS2* gene locus. The three reading frames are shown below the nucleotide sequence. The deduced amino acid sequence of reading frame "a" is provided and contains 909 amino acids. The methionine encoded by the ATG start codon is circled in open reading frame "a" of Fig. 2. The A of the ATG start codon is nucleotide 31 of Fig. 2.

Fig. 3 is the nucleotide sequence of the *avrRpt2* gene and its deduced amino acid sequence. A potential ribosome binding site is underlined. An inverted repeat is indicated by horizontal arrows at the 3' end of the open reading frame. The deduced amino acid sequence is provided below the nucleotide sequence of the open reading frame.

Fig. 4 is a schematic summary of the complementation analysis that allowed functional confirmation that the DNA carried on p4104 and p4115 (encoding cDNA-4) confers *RPS2* disease resistance activity to *Arabidopsis thaliana* plants previously lacking *RPS2* disease resistance activity. Small vertical marks along the "genome" line represent restriction enzyme *Eco*RI recognition sites, and the numbers above this line represent the size, in kilobase pairs (kb), of the resulting DNA fragments (see also Fig. 1E). Opposite "cDNAs" are the approximate locations of the coding sequences for RNA transcripts (See also Fig. 1F); arrowheads indicate the direction of transcription for cDNAs 4, 5, and 6. For functional complementation experiments, *rps2-201C/rps2-201C* plants were genetically transformed with the

Arabidopsis thaliana genomic DNA sequences indicated; these sequenc s were carried on the named plasmids (derivatives of the binary cosmid vector pSLJ4541) and delivered to the plant via *Agrobacterium*-mediated transformation methods. The disease resistance phenotype of the resulting transformants following inoculation with *P. syringae* expressing *avrRpt2* is given as "Sus." (susceptible, no resistance response) or "Res." (disease resistant).

Fig. 5A shows regions of sequence similarity between the L-6 protein of flax, N protein of tobacco, Prf protein of tomato, and rps2 protein of *Arabidopsis*.

Fig. 5B shows sequence similarity between the N and L-6 proteins.

Fig. 6 shows a sequence analysis of RPS2 polypeptide showing polypeptide regions corresponding to an N-terminal hydrophobic region, a leucine zipper, NBSs (kinase-1a, kinase-2, and kinase-3 motifs), and a predicted membrane integrated region.

Fig. 7 shows the amino acid sequence of the RPS2 LRR (amino acids 505-867). The top line indicates the consensus sequences for the *RPS2* LRR. An "X" stands for an arbitrary amino acid sequence and an "a" stands for an aliphatic amino acid residue. The consensus sequence for the *RPS2* LRR is closely related to the consensus for the yeast adenylate cyclase CYR1 LRR (PX Xa XXL XXL XXLXL XXNXaXXa). The amino acid residues that match the consensus sequence are shown in bold. Although this figure shows 14 LRRs, the C-terminal boundary of the LRR is not very clear because the LRR closer to the C-terminus does not fit the consensus sequence very well.

Fig. 8 shows a sequence analysis of RPS2, indicating regions with similarity to leucine zipper, P-loop, membrane-spanning, and leucine-rich repeat motifs. Regions with

similarity to defined functional domains are indicated with a line over the relevant amino acids. Potential N-glycosylation sequences are marked with a dot, and the location of the rps2-201 Thr to Pro mutation at animo acid 668 is marked with an asterisk.

Fig. 9 is a schematic representation of the transient assay method. The top panel shows the essential principles of the assay. The bottom panel shows a schematic representation of the actual transient assay procedure. *Psp* NP53121 is used because it is a weak *Arabidopsis* pathogen, but potent in causing the HR when carrying an avirulence gene. In the absence of an HR, the damage to plant cells infected with NP53121 is minimal, enhancing the difference of GUS accumulation in cells that undergo the HR in comparison to those that do not. Prior to bombardment, one half of an *Arabidopsis* leaf is infiltrated with P. syringae (stippled side of leaf); the other half of the leaf serves as a noninfected control, an "internal" reference for the infected side, and as a measure of transformation efficiency.

Fig. 10, panels A-B, are photographs showing the complementation of the rps2 mutant phenotype using the biolistic transient expression assay. The left sides of rps2-101C mutant leaves were infiltrated with Psp 3121/avrRpt2. Infiltrated leaves were cobombarded with either 35S-uidA plus ΔGUS (Panel A) or 35S-uidA plus 35S-RPS2 (cDNA-2 clone 4) (Panel B). Note that in Panel B the infected side of the leaf shows less GUS activity than the uninfected side, indicating that the transformed cells on the infected side underwent an HR and that 35S-RPS2 complemented the mutant phenotype (see Fig. 9).

Fig. 11 is a schematic representation of pKEx4tr showing the structure of this cDNA expression vector. For

convenience, the multiple cloning site contains the 8bp recognition sequences for PmeI and NotI and is flanked by T7 and T3 promoters. The region spanning the modified 35S promoter to the nopaline synthase 3' sequences (nos 3') was cloned into the Hind III-EcoRI site of pUC18, resulting in the loss of the EcoRI site.

Fig. 12 shows a nucleic acid sequence of the tomato Prf gene.

The Genetic Basis for Resistance to Pathogens

An overview of the interaction between a plant host and a microbial pathogen is presented. The invasion of a plant by a potential pathogen can have a range of outcomes delineated by the following outcomes: either the pathogen successfully proliferates in the host, causing associated disease symptoms, or its growth is halted by the host defenses. In some plant-pathogen interactions, the visible hallmark of an active defense response is the so-called hypersensitive response or "HR". The HR involves rapid necrosis of cells near the site of the infection and may include the formation of a visible dry brown lesion. Pathogens which elicit an HR on a given host are said to be avirulent on that host, the host is said to be resistant, and the plant-pathogen interaction is said to be incompatible. Strains which proliferate and cause disease on a particular host are said to be virulent; in this case the host is said to be susceptible, and the plant-pathogen interaction is said to be compatible

"Classical" genetic analysis has been used successfully to help elucidate the genetic basis of plant-pathogen recognition for those cases in which a series of strains (races) of a particular fungal or bacterial pathogen are either virulent or avirulent on a series of cultivars

(or different wild accessions) of a particular host species. In many such cases, genetic analysis of both the host and the pathogen revealed that many avirulent fungal and bacterial strains differ from virulent ones by the possession of one or more avirulence (*avr*) genes that have corresponding "resistance" genes in the host. This avirulence gene-resistance gene correspondence is termed the "gene-for-gene" model (Crute, et al., (1985) pp 197-309 in: *Mechanisms of Resistance to Plant Disease*. R.S.S. Fraser, ed.; Ellingboe, (1981) Annu. Rev. Phytopathol. 19:125-143; Flor, (1971) Annu. Rev. Phytopathol. 9:275-296; Keen and Staskawicz, (1988) supra; and Keen et al. in: *Application of Biotechnology to Plant Pathogen Control*. I. Chet, ed., John Wiley & Sons, 1993, pp. 65-88). According to a simple formulation of this model, plant resistance genes encode specific receptors for molecular signals generated by *avr* genes. Signal transduction pathway(s) then carry the signal to a set of target genes that initiate the HR and other host defenses (Gabriel and Rolfe, (1990) Annu. Rev. Phytopathol. 28:365-391). Despite this simple predictive model, the molecular basis of the *avr*-resistance gene interaction is still unknown.

One basic prediction of the gene-for-gene hypothesis has been convincingly confirmed at the molecular level by the cloning of a variety of bacterial *avr* genes (Innes, et al., (1993) J. Bacteriol. 175:4859-4869; Dong, et al., (1991) Plant Cell 3:61-72; Whelan et al., (1991) Plant Cell 3:49-59; Staskawicz et al., (1987) J. Bacteriol. 169:5789-5794; Gabriel et al., (1986) P.N.A.S., USA 83:6415-6419; Keen and Staskawicz, (1988) Annu. Rev. Microbiol. 42:421-440; Kobayashi et al., (1990) Mol. Plant-Microbe Interact. 3:94-102 and (1990) Mol. Plant-Microbe Interact. 3:103-111). Many of these cloned avirulence genes have been shown to

correspond to individual resistance genes in the cognate host plants and have been shown to confer an avirulent phenotype when transferred to an otherwise virulent strain. The *avrRpt2* locus was isolated from *Pseudomonas syringae* pv. *tomato* and sequenced by Innes et al. (Innes, R. et al. (1993) J. Bacteriol. 175:4859-4869). Fig. 3 is the nucleotide sequence and deduced amino acid sequence of the *avrRpt2* gene.

Examples of known signals to which plants respond when infected by pathogens include harpins from *Erwinia* (Wei et al. (1992) Science 257:85-88) and *Pseudomonas* (He et al. (1993) Cell 73:1255-1266); avr4 (Joosten et al. (1994) Nature 367:384-386) and avr9 peptides (van den Ackerveken et al (1992) Plant J. 2:359-366) from *Cladosporium*; *PopA1* from *Pseudomonas* (Arlat et al. (1994) EMBO J. 13:543-553); avrD-generated lipopolysaccharide (Midland et al. (1993) J. Org. Chem. 58:2940-2945); and *NIP1* from *Rhynchosporium* (Hahn et al. (1993) Mol. Plant-Microbe Interact. 6:745-754).

Compared to *avr* genes, considerably less is known about plant resistance genes that correspond to specific *avr*-generated signals. The plant resistance gene, *RPS2* (rps for resistance to Pseudomonas syringae), the first gene of a new, previously unidentified class of plant disease resistance genes corresponds to a specific *avr* gene (*avrRpt2*). Some of the work leading up to the cloning of *RPS2* is described in Yu, et al., (1993), Molecular Plant-Microbe Interactions 6:434-443 and in Kunkel, et al., (1993) Plant Cell 5:865-875.

An apparently unrelated avirulence gene which corresponds specifically to plant disease resistance gene, *Pto*, has been isolated from tomato (*Lycopersicon esculentum*) (Martin et al., (1993) Science 262:1432-1436). Tomato plants expressing the *Pto* gene are resistant to

infection by strains of *Pseudomonas syringae* pv. *tomato* that express the *avrPto* avirulence gene. The amino acid sequence inferred from the *Pto* gene DNA sequence displays strong similarity to serine-threonine protein kinases, implicating *Pto* in signal transduction. No similarity to the tomato *Pto* locus or any known protein kinases was observed for *RPS2*, suggesting that *RPS2* is representative of a new class of plant disease resistance genes.

The isolation of a race-specific resistance gene from *Zea mays* (corn) known as *Hm1* has been reported (Johal and Briggs (1992) Science 258:985-987). *Hm1* confers resistance against specific races of the fungal pathogen *Cochliobolus carbonum* by controlling degradation of a fungal toxin, a strategy that is mechanistically distinct from the avirulence-gene specific resistance of the *RPS2-avrRpt2* resistance mechanism.

The cloned *RPS2* gene of the invention can be used to facilitate the construction of plants that are resistant to specific pathogens and to overcome the inability to transfer disease resistance genes between species using classical breeding techniques (Keen et al., (1993), supra). There now follows a description of the cloning and characterization of an *Arabidopsis thaliana RPS2* genetic locus, the *RPS2* genomic DNA, and the *RPS2* cDNA. The *avrRpt2* gene and the *RPS2* gene, as well as mutants *rps2-101C*, *rps2-102C*, and *rps2-201C* (also designated *rps2-201*), are described in Dong, et al., (1991) Plant Cell 3:61-72; Yu, et al., (1993) supra; Kunkel et al., (1993) supra; Whalen et al., (1991), supra; and Innes et al., (1993), supra). A mutant designated *rps2-101N* has also been isolated. The identification and cloning of the *RPS2* gene is described below.

RPS2 Overcomes Sensitivity to Pathogens Carrying the avrRpt2 Gene

To demonstrate the genetic relationship between an avirulence gene in the pathogen and a resistance gene in the host, it was necessary first to isolate an avirulence gene. By screening *Pseudomonas* strains that are known pathogens of crop plants related to *Arabidopsis*, highly virulent strains, *P. syringae* pv. *maculicola (Psm)* ES4326, *P. syringae* pv. *tomato* (*Pst*) DC3000, and an avirulent strain, *Pst* MM1065 were identified and analyzed as to their respective abilities to grow in wild type *Arabidopsis thaliana* plants (Dong *et al.*, (1991) Plant Cell, 3:61-72; Whalen et al., (1991) Plant Cell 3:49-59; MM1065 is designated JL1065 in Whalen et al.). *Psm* ES4326 or *Pst* DC3000 can multiply 10^4 fold in *Arabidopsis thaliana* leaves and cause water-soaked lesions that appear over the course of two days. *Pst* MM1065 multiplies a maximum of 10 fold in *Arabidopsis thaliana* leaves and causes the appearance of a mildly chlorotic dry lesion after 48 hours. Thus, disease resistance is associated with severely inhibited growth of the pathogen.

An avirulence gene (*avr*) of the *Pst* MM1065 strain was cloned using standard techniques as described in Dong *et al.* (1991), Plant Cell 3:61-72; Whalen et al., (1991) supra; and Innes et al., (1993), supra. The isolated avirulence gene from this strain was designated *avrRpt2*. Normally, the virulent strain *Psm* ES4326 or *Pst* DC3000 causes the appearance of disease symptoms after 48 hours as described above. In contrast, *Psm* ES4326/*avrRpt2* or *Pst* DC3000/*avrRpt2* elicits the appearance of a visible necrotic hypersensitivity response (HR) within 16 hours and multiplies 50 fold less than *Psm* ES4326 or *Pst* DC3000 in wild type *Arabidopsis thaliana* leaves (Dong *et al.*, (1991),

supra; and Whalen et al., (1991), supra). Thus, disease resistance in a wild type *Arabidopsis* plant requires, in part, an avirulence gene in the pathogen or a signal generated by the avirulence gene.

The isolation of four *Arabidopsis thaliana* disease resistance mutants has been described using the cloned *avrRpt2* gene to search for the host gene required for disease resistance to pathogens carrying the *avrRpt2* gene (Yu et al., (1993), supra; Kunkel et al., (1993), supra). The four *Arabidopsis thaliana* mutants failed to develop an HR when infiltrated with *Psm* ES4326/*avrRpt2* or *Pst* DC3000/*avrRpt2* as expected for plants having lost their disease resistance capacity. In the case of one of these mutants, approximately 3000 five to six week old M_2 ecotype Columbia (Col-0 plants) plants generated by ethyl methanesulfonic acid (EMS) mutagenesis were hand-inoculated with *Psm* ES4326/*avrRpt2* and a single mutant, *rps2-101C*, was identified (resistance to Pseudomonas syringae) (Yu *et al.*, (1993), supra).

The second mutant was isolated using a procedure that specifically enriches for mutants unable to mount an HR (Yu *et al.*, (1993), supra). When 10-day old *Arabidopsis thaliana* seedlings growing on petri plates are infiltrated with *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) NPS3121 versus *Psp* NPS3121/*avrRpt2*, about 90% of the plants infiltrated with *Psp* NPS3121 survive, whereas about 90%-95% of the plants infiltrated with *Psp* NPS3121/*avrRpt2* die. Apparently, vacuum infiltration of an entire small *Arabidopsis thaliana* seedling with *Psp* NPS3121/*avrRpt2* elicits a systemic HR which usually kills the seedling. In contrast, seedlings infiltrated with *Psp* NPS3121 survive because *Psp* NPS3121 is a weak pathogen on *Arabidopsis*

thaliana. The second disease resistance mutant was isolated by infiltrating 4000 EMS-mutagenized Columbia M_2 seedlings with *Psp* NPS3121/*avrRpt2*. Two hundred survivors were obtained. These were transplanted to soil and re-screened by hand inoculation when the plants reached maturity. Of these 200 survivors, one plant failed to give an HR when hand-infiltrated with *Psm* ES4326/*avrRpt2*. This mutant was designated *rps2-102C* (Yu *et al.*, (1993), supra).

A third mutant, *rps2-201C*, was isolated in a screen of approximately 7500 M_2 plants derived from seed of *Arabidopsis thaliana* ecotype Col-0 that had been mutagenized with diepoxybutane (Kunkel et al., (1993), supra). Plants were inoculated by dipping entire leaf rosettes into a solution containing *Pst* DC3000/*avrRpt2* bacteria and the surfactant Silwet L-77 (Whalen et al., (1991), supra), incubating plants in a controlled environment growth chamber for three to four days, and then visually observing disease symptom development. This screen revealed four mutant lines (carrying the *rps2-201C*, *rps2-202C*, *rps2-203C*, and *rps2-204C* alleles), and plants homozygous for *rps2-201C* were a primary subject for further study (Kunkel et al., (1993), supra and the instant application).

Isolation of the fourth *rps2* mutant, *rps2-101N*, has not yet been published. This fourth isolate is either a mutant or a susceptible *Arabidopsis* ecotype. Seeds of the *Arabidopsis* Nossen ecotype were gamma-irradiated and then sown densely in flats and allowed to germinate and grow through a nylon mesh. When the plants were five to six weeks old, the flats were inverted, the plants were partially submerged in a tray containing a culture of *Psm* ES4326/*avrRpt2*, and the plants were vacuum infiltrated in a vacuum desiccator. Plants inoculated this way develop an HR

within 24 hours. Using this procedure, approximately 40,000 plants were screened and one susceptible plant was identified. Subsequent RFLP analysis of this plant suggested that it may not be a Nossen mutant but rather a different *Arabidopsis* ecotype that is susceptible to *Psm* ES4326/*avrRpt2*. This plant is referred to as *rps2-101N*. The isolated mutants *rps2-101C*, *rps2-102C*, *rps2-201C*, and *rps2-101N* are referred to collectively as the "*rps2* mutants".

The *rps2* Mutants Fail to Specifically Respond to the Cloned Avirulence Gene, *avrRpt2*

The *RPS2* gene product is specifically required for resistance to pathogens carrying the avirulence gene, *avrRpt2*. A mutation in Rps2 polypeptide that eliminates or reduces its function would be observable as the absence of a hypersensitive response upon infiltration of the pathogen. The *rps2* mutants displayed disease symptoms or a null response when infiltrated with *Psm* ES4326/*avrRpt2*, *Pst* DC3000/*avrRpt2* or *Psp* NPS3121/*avrRpt2*, respectively. Specifically, no HR response was elicited, indicating that the plants were susceptible and had lost resistance to the pathogen despite the presence of the *avrRpt2* gene in the pathogen.

Pathogen growth in *rps2* mutant plant leaves was similar in the presence and absence of the *avrRpt2* gene. *Psm* ES4326 and *Psm* ES4326/*avrRpt2* growth in *rps2* mutants was compared and found to multiply equally well in the *rps2* mutants, at the same rate that *Psm* Es4326 multiplied in wild-type *Arabidopsis* leaves. Similar results were observed for *Pst* DC3000 and *Pst* DC3000/*avrRpt2* growth in *rps2* mutants.

The *rps2* mutants displayed a HR when infiltrated with *Pseudomonas* pathogens carrying other avr genes, *Psm* ES4326/*avrB*, *Pst* DC3000/*avrB*, *Psm* ES4326/*avrRpm1*, *Pst* DC3000/*avrRpm1*. The ability to mount an HR to an *avr* gene other than *avrRpt2* indicates that the *rps2* mutants isolated by selection with *avrRpt2* are specific to *avrRpt2*.

Mapping and Cloning of the *RPS2* Gene

Genetic analysis of *rps2* mutants *rps2-101C*, *rps2-102C*, *rps-201C* and *rps-101N* showed that they all corresponded to genes that segregated as expected for a single Mendelian locus and that all four were most likely allelic. The four *rps2* mutants were mapped to the bottom of chromosome IV using standard RFLP mapping procedures including polymerase chain reaction (PCR)-based markers (Yu *et al.*, (1993), supra; Kunkel *et al.*, (1993), supra; and Mindrinos, M., unpublished). Segregation analysis showed that *rps2-101C* and *rps2-102C* are tightly linked to the PCR marker, PG11, while the RFLP marker M600 was used to define the chromosome location of the *rps2-201C* mutation (Fig. 1A) (Yu *et al.*, (1993), supra; Kunkel *et al.*, (1993), supra). *RPS2* has subsequently been mapped to the centromeric side of PG11.

Heterozygous *RPS2/rps2* plants display a defense response that is intermediate between those displayed by the wild-type and homozygous *rps2/rps2* mutant plants (Yu, et al., (1993), supra; and Kunkel et al., (1993), supra). The heterozygous plants mounted an HR in response to *Psm* ES4326/*avrRpt2* or *Pst* DC3000/*avrRpt2* infiltration; however, the HR appeared later than in wild type plants and required a higher minimum inoculum (Yu, et al., (1993), supra; and Kunkel et al., (1993), supra).

High Resolution Mapping of the *RPS2* Gene and *RPS2* cDNA Isolation

To carry out map-based cloning of the *RPS2* gene, *rps2-101N/rps2-101N* was crossed with Landsberg *erecta* *RPS2/RPS2*. Plants of the F_1 generation were allowed to self pollinate (to "self") and 165 F_2 plants were selfed to generate F_3 families. Standard RFLP mapping procedures showed that *rps2-101N* maps close to and on the centromeric side of the RFLP marker, PG11. To obtain a more detailed map position, *rps2-101N/rps-101N* was crossed with a doubly marked Landsberg *erecta* strain containing the recessive mutations, *cer2* and *ap2*. The genetic distance between *cer2* and *ap2* is approximately 15 cM, and the *rps2* locus is located within this interval. F_2 plants that displayed either a *CER2 ap2* or a *cer2 AP2* genotype were collected, selfed, and scored for *RPS2* by inoculating at least 20 F_3 plants for each F_2 with *Psm* ES4326/*avrRpt2*. DNA was also prepared from a pool of approximately 20 F_3 plants for each F_2 line. The *CER2 ap2* and *cer2 AP2* recombinants were used to carry out a chromosome walk that is illustrated in Figure 1.

As shown in Figure 1, *RPS2* was mapped to a 28-35 kb region spanned by cosmid clones E4-4 and E4-6. This region contains at least six genes that produce detectable transcripts. There were no significant differences in the sizes of the transcripts or their level of expression in the *rps2* mutants as determined by RNA blot analysis. cDNA clones of each of these transcripts were isolated and five of these were sequenced. As is described below, one of these transcripts, cDNA-4, was shown to correspond to the *RPS2* locus. From this study, three independent cDNA clones (cDNA-4-4, cDNA-4-5, and cDNA-4-11) were obtained

corresponding to *RPS2* from Columbia ecotype wild type plants. The apparent sizes of *RPS2* transcripts were 3.8 and 3.1 kb as determined by RNA blot analysis.

A fourth independent cDNA-4 clone (cDNA-4-2453) was obtained using map-based isolation of *RPS2* in a separate study. Yeast artificial chromosome (YAC) clones were identified that carry contiguous, overlapping inserts of *Arabidopsis thaliana* ecotype Col-O genomic DNA from the M600 region spanning approximately 900 kb in the *RPS2* region. *Arabidopsis* YAC libraries were obtained from J. Ecker and E. Ward, supra and from E. Grill (Grill and Somerville (1991) Mol. Gen. Genet. 226:484-490). Cosmids designated "H" and "E" were derived from the YAC inserts and were used in the isolation of *RPS2* (Fig. 1).

The genetic and physical location of *RPS2* was more precisely defined using physically mapped RFLP, RAPD (random amplified polymorphic DNA) and CAPS (cleaved amplified polymorphic sequence) markers. Segregating populations from crosses between plants of genotype *RPS2/RPS2* (No-O wild type) and *rps2-201/rps2-201* (Col-O background) were used for genetic mapping. The *RPS2* locus was mapped using markers 17B7LE, PG11, M600 and other markers. For high-resolution genetic mapping, a set of tightly linked RFLP markers was generated using insert end fragments from YAC and cosmid clones (Fig. 1) (Kunkel et al. (1993), supra; Konieczny and Ausubel (1993) Plant J. 4:403-410; and Chang et al. (1988) PNAS USA 85:6856-6860). Cosmid clones E4-4 and E4-6 were then used to identify expressed transcripts (designated cDNA-4, -5, -6, -7, -8 of Fig 1F) from this region, including the cDNA-4-2453 clone.

RPS2 DNA Sequence Analysis

DNA sequence analysis of cDNA-4 from wild-type Col-O plants and from mutants *rps2-101C*, *rps2-102C*, *rps2-201C* and *rps2-101N* showed that cDNA-4 corresponds to *RPS2*. DNA sequence analysis of *rps2-101C*, *rps2-102C* and *rps2-201C* revealed changes from the wild-type sequence as shown in Table 1. The numbering system in Table 1 starts at the ATG start codon encoding the first methionine where A is nucleotide 1. DNA sequence analysis of cDNA-4 corresponding to mutant *rps2-102C* showed that it differed from the wild type sequence at amino acid residue 476. Moreover, DNA sequence analysis of the cDNA corresponding to cDNA-4 from *rps2-101N* showed that it contained a 10 bp insertion at amino acid residue 581, a site within the leucine-rich repeat region which causes a shift in the *RPS2* reading frame. Mutant *rps2-101C* contains a mutation that leads to the formation of a chain termination codon. The DNA sequence of mutant allele *rps2-201C* revealed a mutation altering a single amino acid within a segment of the LRR region that also has similarity to the helix-loop-helix motif, further supporting the designation of this locus as the *RPS2* gene. The DNA and amino acid sequences are shown in Figure 2.

Table 1

Mutant	Wild type	position of mutation	Change
rps2-101C	703 TGA 705	704	TAA Stop Codon
rps2-101N	1741 GTG 1743	1741	GTGGAGTTGTATG Insertion
rps2-102C	1426 AGA 1428 arg	1427	AAA Amino acid 476 lys

rps2-201C	2002	ACC thr	2004	2002	CCC pro	Amino acid

DNA sequence analysis of cDNA-4 corresponding to *RPS2* from wild-type Col-0 plants revealed an open reading frame (between two stop codons) spanning 2,751 bp. There are 2,727 bp between the first methionine codon of this reading frame and the 3'-stop codon, which corresponds to a deduced 909 amino acid polypeptide (See open reading frame "a" of Fig. 2). The amino acid sequence has a relative molecular weight of 104,460 and a pI of 6.51.

As discussed below, *RPS2* belongs to a new class of disease resistance genes; the structure of the Rps2 polypeptide does not resemble the protein structure of the product of the previously cloned and publicized avirulence gene-specific plant disease resistance gene, *Pto*, which has a putative protein kinase domain. From the above analysis of the deduced amino acid sequence, *RPS2* contains several distinct protein domains conserved in other proteins from both eukaryotes and prokaryotes. These domains include, but are not limited, to Leucine Rich Repeats (LRR) (Kobe and Deisenhofer, (1994) Nature 366:751-756); nucleotide binding site, e.g. the kinase 1a motif (P-loop) (Saraste et al. (1990) Trends in Biological Sciences TIBS 15:430-434; Helix-Loop-Helix (Murre et al. (1989) Cell 56:777-783; and Leucine Zipper (Rodrigues and Park (1993) Mol. Cell Biol. 13:6711-6722). The amino acid sequence of Rps2 contains a LRR motif (LRR motif from amino acid residue 505 to amino acid residue 867), which is present in many known proteins and which is thought to be involved in protein-protein interactions and may thus allow interaction with other proteins that are involved in plant disease resistance. The N-terminal portion of the Rps2 polypeptide LRR is, for example, related

to the LRR of yeast (*Saccharomyces cerevisiae*) adenylate cyclase, CYR1. A region predicted to be a transmembrane spanning domain (Klein et al. (1985) Biochim., Biophys. Acta 815:468-476) is located from amino acid residue 350 to amino acid residue 365, N-terminal to the LRR. An ATP/GTP binding site motif (P-loop) is predicted to be located between amino acid residue 177 and amino acid residue 194, inclusive. The motifs are discussed in more detail below.

From the above analysis of the deduced amino acid sequence, the Rps2 polypeptide may have a membrane-receptor structure which consists of an N-terminal extracellular region and a C-terminal cytoplasmic region. Alternatively, the topology of the Rps2 may be the opposite: an N-terminal cytoplasmic region and a C-terminal extracellular region. LRR motifs are extracellular in many cases and the Rps2 LRR contains five potential N-glycosylation sites.

Identification of *RPS2* by Functional Complementation

Complementation of *rps2-201* homozygotes with genomic DNA corresponding to *Arabidopsis thaliana* functionally confirmed that the genomic region encoding cDNA-4 carries *RPS2* activity. Cosmids were constructed that contained overlapping contiguous sequences of wild type *Arabidopsis thaliana* DNA from the *RPS2* region contained in YACs EW11D4, EW9C3, and YUP11F1 of Fig. 1 and Fig. 4. The cosmid vectors were constructed from pSLJ4541 (obtained from J. Jones, Sainsbury Institute, Norwich, England) which contains sequences that allow the inserted sequence to be integrated into the plant genome via *Agrobacterium*-mediated transformation (designated "binary cosmid"). "H" and "E" cosmids (Fig. 1) were used to identify clones carrying DNA from the *Arabidopsis thaliana* genomic *RPS2* region.

More than forty binary cosmids containing inserted *RPS2* region DNA were used to transform *rps2-201* homozygous mutants utilizing *Agrobacterium*-mediated transformation (Chang et al. ((1990) p. 28, Abstracts of the Fourth International Conference on *Arabidopsis* Research, Vienna, Austria). Transformants which remained susceptible (determined by methods including the observed absence of an HR following infection to *P. syringae* pv. *phaseolicola* strain 3121 carrying *avrRpt2* and Psp 3121 without *avrRpt2*) indicated that the inserted DNA did not contain functional *RPS2*. These cosmids conferred the "Sus." or susceptible phenotype indicated in Fig. 4. Transformants which had acquired *avrRpt2*-specific disease resistance (determined by methods including the display of a strong hypersensitive response (HR) when inoculated with *Psp* 3121 with *avrRpt2*, but not following inoculation with *Psp* 3121 without *avrRpt2*) suggested that the inserted DNA contained a functional *RPS2* gene capable of conferring the "Res." or resistant phenotype indicated in Fig. 4. Transformants obtained using the pD4 binary cosmid displayed a strong resistance phenotype as described above. The presence of the insert DNA in the transformants was confirmed by classical genetic analysis (the tight genetic linkage of the disease resistance phenotype and the kanamycin resistance phenotype conferred by the cotransformed selectable marker) and Southern analysis. These results indicated that *RPS2* is encoded by a segment of the 18 kb *Arabidopsis thaliana* genomic region carried on cosmid pD4 (Fig. 4).

To further localize the *RPS2* locus and confirm its ability to confer a resistance phenotype on the *rps2-201* homozygous mutants, a set of six binary cosmids containing partially overlapping genomic DNA inserts were tested. The overlapping inserts pD2, pD4, pD14, pD15, pD27, and pD47

were chosen based on the location of the transcription corresponding to the five cDNA clones in the *RPS2* region (Fig. 4). These transformation experiments utilized a vacuum infiltration procedure (Bechtold et al. (1993) C.R. Acad. Sci. Paris 316:1194-1199) for *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformations with cosmids pD2, pD14, pD15, pD39, and pD46 were performed using a root transformation/regeneration protocol (Valveekens et al. (1988), PNAS 85:5536-5540). The results of pathogen inoculation experiments assaying for *RPS2* activity in these transformants is indicated in Fig. 4.

These experiments were further confirmed using a modification of the vacuum filtration procedure. In particular, the procedure of Bechtold et al. (supra) was modified such that plants were grown in peat-based potting soil covered with a screen, primary inflorescences were removed, and plants with secondary inflorescences (approximately 3 to 15 cm in length) were inverted directly into infiltration medium, infiltrated, and then grown to seed harvest without removal from soil (detailed protocol available on the AAtDB computer database (43). The presence of introduced sequences in the initial pD4 transformant was verified by DNA blot analysis with a pD4 vector and insert sequences (separately) as probes. The presence of the expected sequences in transformants obtained with the vacuum infiltration protocol was also confirmed by DNA blot analysis. Root transformation experiments (19) were performed with an easily regenerable *rps2-201/rps2-201* x No-0 mapping population. Transformants were obtained for pD4 with in plant transformation, for pD2, 14, 16, 39, and 49 with root transformation, and for pD2, 4, 14, 15, 27, and 47 with vacuum infiltration as modified.

Additional transformation experiments utilized binary cosmids carrying the complete coding region and more than 1 kb of upstream genomic sequence for only cDNA-4 or cDNA-6. Using the vacuum infiltration transformation method, three independent transformants were obtained that carried the wild-type cDNA-6 genomic region in a *rps2-201c* homozygous background (pAD431 of Fig. 4). None of these plants displayed *avrRpt2*-dependent disease resistance. Homozygous *rps2-201c* mutants were transformed with wild-type genomic cDNA-4 (p4104 and p4115, each carrying Col-0 genomic sequences corresponding to all of the cDNA-4 open reading frame, plus approximately 1.7 kb of 5' upstream sequence and approximately 0.3 kb of 3' sequence downstream of the stop codon). These p4104 and p4115 transformants displayed a disease resistance phenotype similar to the wild-type *RPS2* homozygotes from which the *rps2* were derived. Additional mutants (*rps2-101N* and *rps2-101C* homozygotes) also displayed *avrRpt2*-dependent resistance when transformed with the cDNA-4 genomic region.

RPS2 Sequences Allow Detection of Other Resistance Genes

DNA blot analysis of *Arabidopsis thaliana* genomic DNA using *RPS2* cDNA as the probe showed that *Arabidopsis* contains several DNA sequences that hybridize to *RPS2* or a portion thereof, suggesting that there are several related genes in the *Arabidopsis* genome.

From the aforementioned description and the nucleic acid sequence shown in Fig. 2, it is possible to isolate other plant disease resistance genes having about 50% or greater sequence identity to the *RPS2* gene. Detection and isolation can be carried out with an oligonucleotide probe containing the *RPS2* gene or a portion thereof greater than 9 nucleic acids in length, and preferably greater than about

18 nucleic acids in length. Probes to sequences encoding specific structural features of the Rps2 polypeptide are preferred as they provide a means of isolating disease resistance genes having similar structural domains. Hybridization can be done using standard techniques such as are described in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, (1989).

For example, high stringency conditions for detecting the *RPS2* gene include hybridization at about 42°C, and about 50% formamide; a first wash at about 65°C, about 2X SSC, and 1% SDS; followed by a second wash at about 65°C and about 0.1% x SSC. Lower stringency conditions for detecting *RPS* genes having about 50% sequence identity to the *RPS2* gene are detected by, for example, hybridization at about 42°C in the absence of formamide; a first wash at about 42°C, about 6X SSC, and about 1% SDS; and a second wash at about 50°C, about 6X SSC, and about 1% SDS. An approximately 350 nucleotide DNA probe encoding the middle portion of the LRR region of Rps2 was used as a probe in the above example. Under lower stringency conditions, a minimum of 5 DNA bands were detected in *Bam*HI digested *Arabidopsis thaliana* genomic DNA as sequences having sufficient sequence identity to hybridize to DNA encoding the middle portion of the LRR motif of Rps2. Similar results were obtained using a probe containing a 300 nucleotide portion of the *RPS2* gene encoding the extreme N-terminus of Rps2 outside of the LRR motif.

Isolation of other disease resistance genes is performed by PCR amplification techniques well known to those skilled in the art of molecular biology using oligonucleotide primers designed to amplify only sequences flanked by the oligonucleotides in genes having sequence identity to *RPS2*. The primers are optionally designed to

allow cloning of the amplified product into a suitable vector.

The RPS Disease-Resistance Gene Family

As discussed above, we have discovered that the *Arabidopsis RPS2* gene described herein is representative of a new class of plant resistance genes. Analysis of the derived amino acid sequence for *RPS2* revealed several regions of similarity with known polypeptide motifs (see, e.g., Schneider et al., Genes Dev. 6:797 (1991)). Most prominent among these is a region of multiple, leucine-rich repeats (LRRs). The LRR motif has been implicated in protein-protein interactions and ligand binding in a diverse array of proteins (see, e.g., Kornfield et al., Annu. Rev. Biochem. 64:631 (1985); Alber, Curr. Opin. Gen. Dev. 2:205 (1992); Lupas et al., Science 252:116 2 (1991); Saraste et al., Trend Biochem. Sci. 15:430 (1990)). In one example, LRRs form the hormone binding sites of mammalian gonadotropin hormone receptors (see, e.g, Lupas et al., Science 252:1162 (1991)) and, in another example, a domain of yeast adenylate cyclase that interacts with the RAS2 protein (Kornfield et al., Annu. Rev. Biochem. 64:631 (1985)). In *RPS2*, the LRR domain spans amino acids 503-867 and contains fourteen repeat units of length 22-26 amino acids. A portion of each repeat resembles the LRR consensus sequence (I/L/V)XXLXXLXX(I/L)XL. In Figure 7, the LRRs from *RPS2* are shown, as well as an *RPS2* consensus sequence. Within the *RPS2* LRR region, five (of six) sequences matching the N-glycosylation consensus sequence [NX(S/T)] were observed (Figure 8, marked with a dot). In particular, N-glycosylation is predicted to occur at amino acids 158, 543, 666, 757, 778, 787. Interestingly, the single nucleotide difference between functional *RPS2* and

mutant allele *rps2-201* is within the LRR coding region, and this mutation disrupts one of the potential glycosylation sites.

Also observed in the deduced amino acid sequence for *RPS2* is a second potential protein-protein interaction domain, a leucine zipper (see, e.g., von Heijne, J. Mol. Biol. 225:487 (1992)), at amino acids 30-57. This region contains four contiguous heptad repeats that match the leucine zipper consensus sequence (I/R)XDLXXX. Leucine zippers facilitate the dimerization of transcription factors by formation of coiled-coil structures, but no sequences suggestive of an adjacent DNA binding domain (such as a strongly basic region or a potential zinc-finger) were detected in *RPS2*. Coiled-coil regions also promote specific interactions between proteins that are not transcription factors (see, e.g., Ward et al., Plant Mol. Biol. 14:561 (1990); Ecker, Methods 1:186 (1990); Grill et al., Mol. Gen. Genet. 226:484 (1991)), and computer database similarity searches with the region spanning amino acids 30-57 of *RPS2* revealed highest similarity to the coiled-coil regions of numerous myosin and paramyosin proteins.

A third *RPS2* motif was found at the sequence GPGGVGKT at deduced amino acids 182-189. This portion of *RPS2* precisely matches the generalized consensus for the phosphate-binding loop (P-loop) of numerous ATP- and GTP-binding proteins (see, e.g., Saraste et al., supra)). The postulated *RPS2* P-loop is similar to those found in RAS proteins and ATP synthase β-subunits (Saraste et al., supra), but surprisingly is most similar to the published P-loop sequences for the *nifH* and *chvD* genes, respectively. The presence of this P-loop sequence strongly suggests nucleotide triphosphate binding as one aspect of *RPS2* function. This domain is also referred to as a kinase-1a

motif (or a nucleotide binding site, or NBS). Other conserved NBSs are present in the *RPS2* sequence; these NBSs include a kinase-2 motif at amino acids 258-262 and a kinase-3a motif at amino acids 330-335.

Finally, inspection of the *RPS2* sequence reveals a fourth *RPS2* motif, a potential membrane-spanning domain located at amino acids 340-360. Within this region, a conserved GLPLAL motif is found at amino acids 347-352. The presence of the membrane-spanning domain raises the possibility that the *RPS2* protein is membrane localized, with the N-terminal leucine zipper and P-loop domains residing together on the opposite side of the membrane from the LRR region. An orientation in which the C-terminal LRR domain is extracellular is suggested by the fact that five of the six potential N-linked glycosylation sites occur C-terminal to the proposed membrane-spanning domain, as well as by the overall more positive charge of the N-terminal amino acid residues (see, e.g., Kornfield et al., supra; von Heijne, supra). A number of proteins that contain LRRs are postulated or known to be membrane-spanning receptors in which the LRRs are displayed extracellularly as a ligand-binding domain (see, e.g., Lopez et al., Proc. Natl. Acad. Sci. 84:5615 (1987); Braun et al., EMBO J. 10:1885 (1991); Schneider et al., supra).

The plant kingdom contains hundreds of resistance genes that are necessarily divergent since they control different resistance specificities. However, plant defense responses such as production of activated oxygen species, PR-protein gene expression, and the hypersensitive response are common to diverse plant-pathogen interactions. This implies that there are points of convergence in the defense signal transduction pathways downstream of initial pathogen recognition, and also suggests that similar functional

motifs may exist among diverse resistance gene products. Indeed, *RPS2* is dissimilar from previously described disease resistance genes such as *Hm1* or *Pto* (see, e.g., Johal et al., supra; Martin et al., supra), and thus represents a new class of genes having disease resistance capabilities.

Isolation of Other Members of the RPS Disease-Resistance Gene Family Using Conserved Motif Probes and Primers

We have discovered that the *RPS2* motifs described above are conserved in other disease-resistance genes, including, without limitation, the N protein, the L6 protein, and the Prf protein. As shown in Fig. 5(A and B), we have determined that the L6 polypeptide of flax, the N polypeptide of tobacco, and the Prf polypeptide of tomato each share unique regions of similarity (including, but not limited to, the leucine-rich repeats, the membrane-spanning domain, the leucine zipper, and the P-loop and other NBS domains).

On the basis of this discovery, the isolation of virtually any member of the RPS gene family is made possible using standard techniques. In particular, using all or a portion of the amino acid sequence of a conserved RPS motif (for example, the amino acid sequences defining any RPS P-loop, NBS, leucine-rich repeat, leucine zipper, or membrane-spanning region), one may readily design RPS oligonucleotide probes, including RPS degenerate oligonucleotide probes (i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be based upon the sequence of either strand of the DNA comprising the motif. General methods for designing and preparing such probes are provided, for example, in Ausubel et al., supra and *Guide to Molecular Cloning Techniques*, 1987, S. L. Berger and A. R. Kimmel, eds., Academic Press,

New York. These oligonucleotides are useful for RPS gene isolation, either through their use as probes capable of hybridizing to RPS complementary sequences or as primers for various polymerase chain reaction (PCR) cloning strategies.

Hybridization techniques and procedures are well known to those skilled in the art and are described, for example, in Ausubel et al., supra and *Guide to Molecular Cloning Techniques*, 1987, S. L. Berger and A. R. Kimmel, eds., Academic Press, New York. If desired, a combination of different oligonucleotide probes may be used for the screening of the recombinant DNA library. The oligonucleotides are labelled with ^{32}P using methods known in the art, and the detectably-labelled oligonucleotides are used to probe filter replicas from a recombinant plant DNA library. Recombinant DNA libraries may be prepared according to methods well known in the art, for example, as described in Ausubel et al., supra. Positive clones may, if desired, be rescreened with additional oligonucleotide probes based upon other RPS conserved regions. For example, an RPS clone identified based on hybridization with a P-loop-derived probe may be confirmed by re-screening with a leucine-rich repeat-derived oligonucleotide.

As discussed above, RPS oligonucleotides may also be used as primers in PCR cloning strategies. Such PCR methods are well known in the art and described, for example, in PCR Technology, H.A. Erlich, ed., Stockton Press, London, 1989; PCR Protocols: A Guide to Methods and Applications, M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, eds., Academic Press, Inc., New York, 1990; and Ausubel et al., supra. If desired, members of the RPS disease-resistance gene family may be isolated using the PCR "RACE" technique, or Rapid Amplification of cDNA Ends (see, e.g., Innis et

al., supra). By this method, oligonucleotide primers based on an RPS conserved domain are oriented in the 3' and 5' directions and are used to generate overlapping PCR fragments. These overlapping 3'- and 5'-end RACE products are combined to produce an intact full-length cDNA. This method is described in Innis et al., supra; and Frohman et al., Proc. Natl. Acad. Sci. 85:8998, 1988.

Any number of probes and primers according to the invention may be designed based on the conserved RPS motifs described herein. Preferred motifs are boxed in the sequences shown in Fig. 5(A or B). In particular, oligonucleotides according to the invention may be based on the conserved P-loop domain, the amino acids of which are shown below:

MOTIF 1

L6	G MGGIGKTTTA
N	G MGGVGKTTIA
PrfP	G MPGLGKTTLA
RPS2	G PGGVGKTTLM

From these sequences, appropriate oligonucleotides are designed and prepared using standard methods. Particular examples of RPS oligonucleotides based on the P-loop domain are as follows (N is A, C, T, or G).

Based on MOTIF 1:

5' GGNATGGGNGGNNTNGGNAA(A or G)ACNAC 3'

5' NCGNG(A/T)NGTNA(T/G) (G/A/T)A(T/A)NCGNA 3'

5' GG(T or A)NT(T or G or C)GG(T or A)AA(G or A)AC(T or C or A)AC 3'

5' GGNATGGGNGGNNTNGGNAA(A or G)ACNAC 3'

5' N(G or A) (C or T)N(A or G) (A or G or T)NGTNGT(C or T)TTNCCNANNCCN(G or L) (G or C)N(G or A) (T or

G)NCC 3'

5' GGN(C or A)(T or C)N(G or C)(G or C)NGGNNTNGGNAA
(A or G)ACNAC 3'

Other conserved *RPS* motifs useful for oligonucleotide design are shown below. These motifs are also depicted in the sequence of Fig. 5(A or B).

<u>MOTIF 2</u>	
L6	FKILVV LDDVD
N	KKVLIV LDDID
PrfP	KRFLIL IDDVW
RPS2	KRFLLL LDDVW
<u>MOTIF 3</u>	
L6	SRFIIT SR
N	SRIIIT TR
PrfP	SRIILT TR
RPS2	CKVMFT TR
<u>MOTIF 4</u>	
L6	GLPLTLK V
N	GLPLALK V
PrfP	GLPLSVV L
RPS2	GLPLALI T
<u>MOTIF 5</u>	
L6	KISYDAL
N	KISYDGL
PrfP	GFSYKNL
RPS2	KFSYDNL

From the above motifs and the sequence motifs designated in Figure 5A and B, appropriate oligonucleotides are designed

and prepared. Particular examples of such RPS oligonucleotides are as follows (N is A, T, C, or G).

Based on MOTIF 2:

5' T(T or C)GA(T or C)GA(T or C)(A or G)T(T or G or C)(T or G)(A or G)(T or G or C)(G or A)A 3'

5' T(T or C)CCA(G or C or A)A(T or C)(G or A)TC(A or G)TCNA 3'

5' (C or G or A)(T or C)(C or A)NA(T or C)(G or A)TC(G or A)TCNA(G or A or T)NA(G or A or C)NANNA(G or A)NA 3'

5' (T or A)(T or A)N(A or C)(A or G)(A or G)(T or G or A)TN(T or C)TNNTN(G or T or C)TN(A or T or C)TNGA(T or C)GA 3'

Based on MOTIF 3:

5' NCGNG(A or T)NGTNA(T or G)(G or A or T)A(T or A)NCGNGA 3'

5' NCGNG(A or T)NGTNA(T or G)(G or A or T)A(T or A)NCGNGA 3'

5' NC(G or T)N(G or C)(A or T)NGTNA(A or G or T)(A or G or T)AT(A or G or T)AATNG 3'

Based on MOTIF 4:

5' NA(G or A)NGGNA(G or A)NCC 3'

5' GG(T or A)(T or C)T(T or G or C)CC(T or A)(T or C)T(T or G or C)GC(T or C or A)(T or C)T 3'

5' A(A or G)(T or G or A)GC(G or C or A)A(G or A)(T or A)GG(G or C or A)A(G or A)(A or G or T or C)CC 3'

5' NA(G or A)NGGNA(G or A)NCC 3'

5' N(A or G)NN(T or A)(T or C)NA(G or C or A)N(C or G)(A or T or C)NA(G or A)NGGNA(G or A)NCC 3'

5' GGN(T or C)TNCCN(T or C)TN(G or A or T)(C or G)N(T or G or C)T 3'

Based on MOTIF 5:

5' A(A or G)(A or G)TT(A or G)TC(A or G)TA(G or A or T)(G or C)(T or A)(G or A)A(T or A)(C or T)TT 3'

5' A(G or A)N(T or C)(T or C)NT(C or T)(A or G)TAN(G or C)(A or G)NANN(C or T)(C or T) 3'

5' (G or A)(G or A)N(A or T)T(A or C or T)(T or A)(G or C)NTA(T or C)(G or A)AN(A or G)(A or C or G)N(T or C)T 3'

Based on MOTIF 6:

5' GTNTT(T or C)(T or C)TN(T or A)(G or C)NTT(T or C)(A or C)G(A or G)GG 3'

Based on MOTIF 7:

5' CCNAT(A or C or T)TT(T or C)TA(T or C)(G or A)(T or A)(G or T or C)GTNGA(T or C)CC 3'

Based on MOTIF 8:

5' GTNGGNAT(A or C or T)GA(T or C)(G or A)(A or C)NCA 3'

Based on MOTIF 9:

5' (G or A)AA(G or A)CANGC(A or G or T)AT(G or A)TCNA(G or A)(G or A)AA 3'

5' TT(T or C)(T or C)TNGA(T or C)AT(A or C or T)GCNTG(T or C)TT 3'

Based on MOTIF 10:

5' CCCAT(G or A)TC(T or C)(T or C)(T or G)NA(T or G or A)N(T or A)(G or A)(G or A)TC(A or G)TGCAT 3'

5' ATGCA(T or C)GA(T or C)(T or C)(T or A)N(A or C or T)TN(A or C)(A or G)(A or G)GA(T or C)ATGGG 3'

Based on MOTIF 11:

5' NA(G or A)N(G or C)(A or T)(T or C)T(T or C)NA(A or G)(C or T)TT 3'

5' (A or T)(G or C)NAA(A or G)(T or C)TN(A or G)A(A

or G)(A or T)(G or C)N(T or C)T 3'

Based on MOTIF 12:

5' (A or G or T)(A or T)(A or T)(C or T)TCNA(G or A)N(G or C)(A or T)N(T or C)(G or T)NA(G or A)NCC 3'

5' GGN(T or C)TN(A or C)(G or A)N(A or T)(G or L)N(T or C)TNGA 3'

Once a clone encoding a candidate RPS family gene is identified, it is then determined whether such gene is capable of conferring disease-resistance to a plant host using the methods described herein or other methods well known in the art of molecular plant pathology.

A Biolistic Transient Expression Assay For Identification of Plant Resistance Genes

We have developed a functional transient expression system capable of providing a rapid and broadly applicable method for identifying and characterizing virtually any gene for its ability to confer disease-resistance to a plant cell. In brief, the assay system involves delivering by biolistic transformation a candidate plant disease-resistance gene to a plant tissue sample (e.g., a piece of tissue from a leaf) and then evaluating the expression of the gene within the tissue by appraising the presence or absence of a disease-resistance response (e.g., the hypersensitive response). This assay provides a method for identifying disease-resistance genes from a wide variety of plant species, including ones that are not amenable to genetic or transgenic studies.

The principle of the assay is depicted in the top portion of Figure 9. In general, plant cells carrying a mutation in the resistance gene of interest are utilized.

Prior to biolistic transformation, the plant tissue is infiltrated with a phytopathogenic bacterium carrying the corresponding avirulence gene. In addition, a gene to be assayed for its resistance gene activity is co-introduced by biolistics with a reporter gene. The expression of the cobombarded reporter gene serves as an indicator for viability of the transformed cells. Both genes are expressed under the control of a strong and constitutive promoter. If the gene to be assayed does not complement the resistance gene function, the plant cells do not undergo a hypersensitive response (HR) and, therefore, survive (Fig. 9, top panel, right). In this case, cells accumulate a large amount of the reporter gene product. If, on the other hand, a resistance gene is introduced, the plant cells recognize the signal from the avirulence-gene-carrying bacterium and undergo the HR because the expressed resistance gene product complements the function (Fig. 9, top panel, left). In this case, the plant cells do not have enough time to accumulate a large amount of reporter gene product before their death. Given the transformation efficiency estimated by a proper control (such as the uninfected half of the leaf), measuring the accumulation of reporter gene product can thus indicate whether the gene to be assayed complements the resistance gene function.

In one working example, we now demonstrate the effectiveness of the transient expression assay, using the bacterial avirulence gene *avrRpt2* and the corresponding *Arabidopsis thaliana* resistance gene *RPS2* (Fig. 9, bottom panel). In brief, *rps2* mutant leaves, preinfected with *P. syringae* carrying *avrRpt2*, were co-bombarded with two plasmids, one of which contained the *RPS2* gene and the other the Escherichia coli *uidA* gene encoding β-glucuronidase (GUS; Jefferson et al., 1986, supra). Both the *RPS2* and

uidA genes are located downstream of the strong constitutive *35S* promoter from cauliflower mosaic virus (Odell et al., infra). If the *35S-RPS2* construct complements the *rps2* mutation, the transformed cells rapidly undergo programmed cell death in response to the *P. syringae* carrying *avrRpt2*, and relatively little GUS activity accumulates. If the *rps2* mutation is not complemented, cell death does not occur and high levels of GUS activity accumulate. These differences in GUS activity are detected histochemically. Because the cDNA library used to identify *RPS2* was constructed in the expression vector pKEx4tr, the *35S-RPS2* cDNA construct in pKEx4tr could be used directly in the transient assay. As shown in Fig. 11, pKEx4tr is a cDNA expression vector designed for the unidirectional insertion of cDNA inserts. Inserted cDNA is expressed under the control of the 355 cauliflower mosaic virus promoter.

Our results are shown in Fig. 9, lower panel. In this experiment, we infected one side of a leaf of an *rps2* mutant plant with *P. syringae* pv. *phaseloicola* 3121 carrying *avrRpt2* (*Psp* 3121/*avrRpt2*). *Psp* 3121 is a weak pathogen of *A. thaliana* and *Psp 3121/avrRpt2* can elicit an HR in a plant carrying the resistance gene *RPS2* (e.g., a wild type plant). Leaves of 5-week-old *Arabidopsis* plants were infiltrated with an appropriate bacterial suspension at a dose of 2 x 10^8/ml by hand infiltration as described (Dong et al., supra). After an incubation period (typically 2-4 hours), the leaves were bombarded using a Bio-Rad PDS-1000/He apparatus (1100 psi) after 2-4 hr of infection. Gold particles were prepared according to the instructions of the manufacturer. For each bombardment, 1.4 μg of pKEx4tr-G, 0.1 μg of a plasmid to be tested, and 0.5 mg of 1 μm gold particles were used. After the bombardment, the leaves were

leaf, transformation efficiency (i.e., density of transformed cells) is similar on both sides of the leaf. If transformed cells on the infected side are rapidly killed, staining of the cells on the infected side is weaker than staining on the uninfected side. When the resistance gene *RPS2* was co-introduced, the transformed cells on the infected side of the leaf showed much weaker staining than ones on the uninfected side (Fig. 10). In contrast, when an unrelated gene was co-introduced, the transformed cells on the infected side showed similar staining intensity to ones on the uninfected side (Fig. 10).

Thus, as summarized in the Table 2, *35S-RPS4* (cDNA 4), but not cDNA-5 or cDNA-6, complemented the HR phenotype of *rps2-101C*. (See Figure 1)

Table 2

Gene Tested	Response (Decreased GUS Activity)[a]
ΔGUS (*35S-uidA* containing internal *uidA* deletion)	-
cDNA-5 (*35S-AB11*)	
cDNA-4 (*35S-RPS2*)	+
cDNA-6 (*35S-CK1*)	

[a]When decreased GUS activity was observed on the infiltrated side of the leaf, the response was scored as plus (Fig. 10).

Both *RPS2* cDNA-4 clones 4 and 11, corresponding to the two RPS2 different transcript sizes, complemented the *rps2* mutant phenotype, indicating that both transcripts encode a functional product. Moreover, *35S-RPS2* also complemented

mutants *rps2-102C*, *rps2-101N*, and *rps2-201C*, further confirming that the *rps2-101C*, *rps2-102C*, *rps2-201C* and *rps2-101N* mutations are all allelic. In short, the cloned *RPS2* gene complemented the *rps2* mutation in this transient expression assay, and complementation by *RPS2* was observed in all four available *rps2* mutant stains.

Next we used the transient assay system to test the specificity of the cloned *RPS2* gene for an *avrRpt2*-generated signal (i.e., the "gene-for-gene" specificity of a *P. syringae* avirulence gene and a corresponding *A. thaliana* resistance gene (*avrRpm1* and *RPM1*, respectively)). This experiment involved the use of an *rps2-101 rpm1* double mutant that cannot mount an HR when challenged with *P. syringae* carrying *avrRpt2* or the unrelated avirulence gene *avrRpm1* (Debener et al., Plant Journal 1:289-302, 1991). As summarized in Table 3, complementation of the *rps2* mutant phenotype by *35S-RPS2* was only observed in the presence of a signal generated by *avrRpt2*, indicating that *RPS2* does not simply sensitize the plant resistance response in a nonspecific manner.

Table 3

avr Gene	Construct Cobombarded with *35S-uidA*	Response[a]
None (vector only)	ΔGUS[b]	-
avrRPt2	ΔGUS	-
avrRpm1	ΔGUS	-
None (vector only)	*35S-RPS2*	-
avrRpt2	*35S-RPS2*	+
avrRpm1	*35S-RPS2*	-

[a]When decreased GUS activity was observed on the infiltrated side of the leaf, the response was scored as plus. (Figure 10, panel B)

[b]ΔGUS is *35S-uidA* containing an internal deletion in the *uidA* gene.

Also as shown in Table 3, the *RPS2* gene complemented the mutant phenotype when leaves were infected with *Psp* 3121/*avrRpt2* but not with *Psp* 3121/*avrRpm1*. Therefore, the *RPS2* gene complemented only the *rps2* mutation; it did not the *rpm1* mutation.

We have also discovered that overexpression of an rps gene family member, e.g., rps2 but not other genes, in the transient assay leads to apparent cell death, obviating the need to know the corresponding avirulence gene for a putative resistance gene that has been cloned.

Using this assay, any plant disease-resistance gene may be identified from a cDNA expression library. In one particular example, a cDNA library is constructed in an expression vector and then introduced as described herein into a plant cultivar or its corresponding mutant plant lacking the resistance gene of interest. Preferably, the cDNA library is divided into small pools, and each pool co-introduced with a reporter gene. If a pool contains a resistance gene clone (i.e., the pool "complements" the resistance gene function), the positive pool is divided into smaller pools and the same procedure is repeated until identification of a single positive clone is ultimately achieved. This approach facilitates the cloning of any resistance gene of interest without genetic crosses or the creation of transgenics.

We now describe the cloning of another member of the RPS gene family, the Prf gene of tomato.

The initial step for the cloning of the Prf gene came from classical genetic analysis which showed that Prf

was tightly linked to the tomato Pto gene (Salmeron et al., The Plant Cell 6:511-520, 1994). This prompted construction of a cosmid contig of 200 kb in length which encompassed the Pto locus. DNA probes from this contig were used to screen a tomato cDNA library constructed using tomato leaf tissue that had been infected with Pst expressing the avrPto avirulence gene as source material. Two classes of cDNAs were identified based on cross-hybridization of clones to each other. While one class corresponded to members of the Pto gene family, the other class displayed no hybridization to Pto family members. Taking the assumption (based on the aforementioned genetic analysis) that Prf might reside extremely close to the Pto gene, cDNAs from the second class were analyzed further as candidate Prf clones. These clones were hybridized to filters containing DNAs from six independent prf mutant lines that had been isolated by diepoxybutane or fast neutron treatment. In one of the fast neutron mutants, the cDNA probe revealed a 1.1 kb deletion in the genomic DNA, suggesting that the cDNA clone might in fact represent Prf. Wild-type DNA corresponding to the deletion was cloned from Prf/Prf tomato. A 5 kb region was sequenced and found to potentially encode a protein containing P-loop and leucine-rich repeat motifs, supporting the hypothesis that this DNA encoded Prf. The corresponding DNA was cloned and sequenced from the fast neutron mutant plant. Sequencing this DNA confirmed the mutation to be a simple 1.1 kb deletion excising DNA between the potential P-loop and leucine-rich repeat coding regions. The gene is expressed based on RT-PCR analysis which has shown that an mRNA is transcribed from this region. The identity of the cloned DNA as the Prf gene is based on both the existence of the deletion mutation and the predicted protein sequence, which reveals patches of strong similarity to other cloned

disease resistance gene products throughout the amino-terminal half (as described herein). A partial sequence of the Prf gene is shown in Figure 12.

RPS Expression in Transgenic Plant Cells and Plants

The expression of the *RPS2* genes in plants susceptible to pathogens carrying *avrRpt2* is achieved by introducing into a plant a DNA sequence containing the *RPS2* gene for expression of the Rps2 polypeptide. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants are available to the public; such vectors are described in, e.g., Pouwels et al., *Cloning Vectors: A Laboratory Manual*, 1985, Supp. 1987); Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989; and Gelvin et al., *Plant Molecular Biology Manual*, Kluwer Academic Publishers, 1990. Typically, plant expression vectors include (1) one or more cloned plant genes under the transcriptional control of 5′ and 3′ regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

An example of a useful plant promoter which could be used to express a plant resistance gene according to the invention is a caulimovirus promoter, e.g., the cauliflower mosaic virus (CaMV) 35S promoter. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not dependent on virtually

encoded proteins. CaMV is a source for both the 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, e.g., Odel et al., Nature 313:810, (1985)). The CaMV promoter is also highly active in monocots (see, e.g., Dekeyser et al., Plant Cell 2:591, (1990); Terada and Shimamoto, Mol. Gen. Genet. 220:389, (1990)).

Other useful plant promoters include, without limitation, the nonpaline synthase promoter (An et al., Plant Physiol. 88:547, (1988)) and the octopine synthase promoter (Fromm et al., Plant Cell 1:977, (1989)).

For certain applications, it may be desirable to produce the *RPS2* gene product or the *avrRpt2* gene product in an appropriate tissue, at an appropriate level, or at an appropriate developmental time. Thus, there are a variety of gene promoters, each with its own distinct characteristics embodied in its regulatory sequences, shown to be regulated in response to the environment, hormones, and/or developmental cues. These include gene promoters that are responsible for (1) heat-regulated gene expression (see, e.g., Callis et al., Plant Physiol. 88: 965, (1988)), (2) light-regulated gene expression (e.g., the pea *rbcS-3A* described by Kuhlemeier et al., Plant Cell 1: 471, (1989); the maize *rbcS* promoter described by Schaffner and Sheen, Plant Cell 3: 997, (1991); or the chlorophyll a/b-binding protein gene found in pea described by Simpson et al., EMBO J. 4: 2723, (1985)), (3) hormone-regulated gene expression (e.g., the abscisic acid responsive sequences from the *Em* gene of wheat described Marcotte et al., Plant Cell 1:969, (1989)), (4) wound-induced gene expression (e.g., of *wunI* described by Siebertz et al., Plant Cell 1: 961, (1989)), or (5) organ-specific gene expression (e.g., of the tuber-specific storage protein gene described by Roshal et al.,

EMBO J. 6:1155, (1987); the 23-kDa zein gene from maize described by Schernthaner et al., EMBO J. 7: 1249, (1988); or the French bean ß-phaseolin gene described by Bustos et al., Plant Cell 1:839, (1989)).

Plant expression vectors may also optionally include RNA processing signals, e.g, introns, which have been shown to be important for efficient RNA synthesis and accumulation (Callis et al., Genes and Dev. 1: 1183, (1987)). The location of the RNA splice sequences can influence the level of transgene expression in plants. In view of this fact, an intron may be positioned upstream or downstream of an Rps2 polypeptide-encoding sequence in the transgene to modulate levels of gene expression.

In addition to the aforementioned 5′ regulatory control sequences, the expression vectors may also include regulatory control regions which are generally present in the 3′ regions of plant genes (Thornburg et al., Proc. Natl Acad. Sci USA 84: 744, (1987); An et al., Plant Cell 1: 115, (1989)). For example, the 3′ terminator region may be included in the expression vector to increase stability of the mRNA. One such terminator region may be derived from the PI-II terminator region of potato. In addition, other commonly used terminators are derived from the octopine or nopaline synthase signals.

The plant expression vector also typically contains a dominant selectable marker gene used to identify the cells that have become transformed. Useful selectable marker genes for plant systems include genes encoding antibiotic resistance genes, for example, those encoding resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetic-deficient strains. Finally, genes encoding herbicide resistance may

be used as selectable markers; useful herbicide resistance genes include the *bar* gene encoding the enzyme phosphinothricin acetyltransferase, which confers resistance to the broad spectrum herbicide Basta® (Hoechst AG, Frankfurt, Germany).

Efficient use of selectable markers is facilitated by a determination of the susceptibility of a plant cell to a particular selectable agent and a determination of the concentration of this agent which effectively kills most, if not all, of the transformed cells. Some useful concentrations of antibiotics for tobacco transformation include, e.g., 75-100 μg/ml (kanamycin), 20-50 μg/ml (hygromycin), or 5-10 μg/ml (bleomycin). A useful strategy for selection of transformants for herbicide resistance is described, e.g., in Vasil I.K., *Cell Culture and Somatic Cell Genetics of Plants*, Vol I, II, III Laboratory Procedures and Their Applications Academic Press, New York, 1984.

It should be readily apparent to one skilled in the field of plant molecular biology that the level of gene expression is dependent not only on the combination of promoters, RNA processing signals and terminator elements, but also on how these elements are used to increase the levels of gene expression.

The above exemplary techniques may be used for the expression of any gene in the RPS family.

Plant Transformation

Upon construction of the plant expression vector, several standard methods are known for introduction of the recombinant genetic material into the host plant for the generation of a transgenic plant. These methods include (1) Agrobacterium-mediated transformation (*A. tumefaciens* or *A.*

rhizogenes) (see, e.g., Lichtenstein and Fuller In: *Genetic Engineering*, vol 6, PWJ Rigby, ed, London, Academic Press, 1987; and Lichtenstein, C.P., and Draper, J,. In: *DNA Cloning*, Vol II, D.M. Glover, ed, Oxford, IRI Press, 1985), (2) the particle delivery system (see, e.g., Gordon-Kamm et al., Plant Cell 2:603, (1990); or BioRad Technical Bulletin 1687, *supra*), (3) microinjection protocols (see, e.g., Green et al., *Plant Tissue and Cell Culture*, Academic Press, New York, 1987), (4) polyethylene glycol (PEG) procedures (see, e.g., Draper et al., Plant Cell Physiol 23:451, (1982); or e.g., Zhang and Wu, Theor. Appl. Genet. 76:835, (1988)), (5) liposome-mediated DNA uptake (see, e.g., Freeman et al., Plant Cell Physiol 25: 1353, (1984)), (6) electroporation protocols (see, e.g., Gelvin et al supra; Dekeyser et al. supra; or Fromm et al Nature 319: 791, (1986)), and (7) the vortexing method (see, e.g., Kindle, K., Proc. Natl. Acad. Sci., USA 87:1228, (1990)).

The following is an example outlining an *Agrobacterium*-mediated plant transformation. The general process for manipulating genes to be transferred into the genome of plant cells is carried out in two phases. First, all the cloning and DNA modification steps are done in *E. coli*, and the plasmid containing the gene construct of interest is transferred by conjugation into *Agrobacterium*. Second, the resulting *Agrobacterium* strain is used to transform plant cells. Thus, for the generalized plant expression vector, the plasmid contains an origin of replication that allows it to replicate in *Agrobacterium* and a high copy number origin of replication functional in *E. coli*. This permits facile production and testing of transgenes in *E.coli* prior to transfer to *Agrobacterium* for subsequent introduction into plants. Resistance genes can be carried on the vector, one for selection in bacteria,

e.g., streptomycin, and the other that will express in plants, e.g., a gene encoding for kanamycin resistance or an herbicide resistance gene. Also present are restriction endonuclease sites for the addition of one or more transgenes operably linked to appropriate regulatory sequences and directional T-DNA border sequences which, when recognized by the transfer functions of *Agrobacterium*, delimit the region that will be transferred to the plant.

In another example, plant cells may be transformed by shooting into the cell tungsten microprojectiles on which cloned DNA is precipitated. In the Biolistic Apparatus (Bio-Rad, Hercules, CA) used for the shooting, a gunpowder charge (22 caliber Power Piston Tool Charge) or an air-driven blast drives a plastic macroprojectile through a gun barrel. An aliquot of a suspension of tungsten particles on which DNA has been precipitated is placed on the front of the plastic macroprojectile. The latter is fired at an acrylic stopping plate that has a hole through it that is too small for the macroprojectile to go through. As a result, the plastic macroprojectile smashes against the stopping plate and the tungsten microprojectiles continue toward their target through the hole in the plate. For the instant invention the target can be any plant cell, tissue, seed, or embryo. The DNA introduced into the cell on the microprojectiles becomes integrated into either the nucleus or the chloroplast.

Transfer and expression of transgenes in plant cells is now routine practice to those skilled in the art. It has become a major tool to carry out gene expression studies and to attempt to obtain improved plant varieties of agricultural or commercial interest.

Transgenic Plant Regeneration

Plant cells transformed with a plant expression vector can be regenerated, e.g., from single cells, callus tissue or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues and organs from almost any plant can be successfully cultured to regenerate an entire plant; such techniques are described, e.g., in Vasil supra; Green et al., supra; Weissbach and Weissbach, supra; and Gelvin et al., supra.

In one possible example, a vector carrying a selectable marker gene (e.g., kanamycin resistance), a cloned *RPS2* gene under the control of its own promoter and terminator or, if desired, under the control of exogenous regulatory sequences such as the 35S CaMV promoter and the nopaline synthase terminator is transformed into *Agrobacterium*. Transformation of leaf tissue with vector-containing *Agrobacterium* is carried out as described by Horsch et al. (Science 227: 1229, (1985)). Putative transformants are selected after a few weeks (e.g., 3 to 5 weeks) on plant tissue culture media containing kanamycin (e.g. 100 μg/ml). Kanamycin-resistant shoots are then placed on plant tissue culture media without hormones for root initiation. Kanamycin-resistant plants are then selected for greenhouse growth. If desired, seeds from self-fertilized transgenic plants can then be sowed in a soil-less media and grown in a greenhouse. Kanamycin-resistant progeny are selected by sowing surfaced sterilized seeds on hormone-free kanamycin-containing media. Analysis for the integration of the transgene is accomplished by standard techniques (see, e.g., Ausubel et al. supra; Gelvin et al. supra).

Transgenic plants expressing the selectable marker are then screened for transmission of the transgene DNA by

standard immunoblot and DNA and RNA detection techniques. Each positive transgenic plant and its transgenic progeny are unique in comparison to other transgenic plants established with the same transgene. Integration of the transgene DNA into the plant genomic DNA is in most cases random and the site of integration can profoundly effect the levels, and the tissue and developmental patterns of transgene expression. Consequently, a number of transgenic lines are usually screened for each transgene to identify and select plants with the most appropriate expression profiles.

Transgenic lines are evaluated for levels of transgene expression. Expression at the RNA level is determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis are employed and include PCR amplification assays using oligonucleotide primers designed to amplify only transgene RNA templates and solution hybridization assays using transgene-specific probes (see, e.g., Ausubel et al., supra). The RNA-positive plants are then analyzed for protein expression by Western immunoblot analysis using Rps2 polypeptide-specific antibodies (see, e.g., Ausubel et al., supra). In addition, *in situ* hybridization and immunocytochemistry according to standard protocols can be done using transgene-specific nucleotide probes and antibodies, respectively, to localize sites of expression within transgenic tissue.

Once the Rps2 polypeptide has been expressed in any cell or in a transgenic plant (e.g., as described above), it can be isolated using any standard technique, e.g., affinity chromatography. In one example, an anti-Rps2 antibody (e.g., produced as described in Ausubel et al., supra, or by any standard technique) may be attached to a column and used

to isolate the polypeptide. Lysis and fractionation of Rps2-producing cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant polypeptide can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, Work and Burdon, eds., Elsevier, 1980).

These general techniques of polypeptide expression and purification can also be used to produce and isolate useful Rps2 fragments or analogs.

Antibody Production

Using a polypeptide described above (e.g., the recombinant protein or a chemically synthesized *RPS* peptide based on its deduced amino acid sequence), polyclonal antibodies which bind specifically to an *RPS* polypeptide may be produced by standard techniques (see, e.g., Ausubel et al., supra) and isolated, e.g., following peptide antigen affinity chromatography. Monoclonal antibodies can also be prepared using standard hybridoma technology (see, e.g., Kohler et al., *Nature* 256: 495, 1975; Kohler et al., *Eur. J. Immunol.* 6: 292, 1976; Hammerling et al., in *Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, N.Y., 1981; and Ausubel et al., supra).

Once produced, polyclonal or monoclonal antibodies are tested for specific *RSP* polypeptide recognition by Western blot or immunoprecipitation analysis (by methods described in Ausubel et al., supra). Antibodies which specifically recognize a *RPS* polypeptide are considered to be useful in the invention; such antibodies may be used, e.g., for screening recombinant expression libraries as

described in Ausubel et al., supra. Exemplary peptides (derived from Rps2) for antibody production include:

LKFSYDNLESDLL

GVYGPGGVGKTTLMQS

GGLPLALITLGGAM

Use

Introduction of *RPS2* into a transformed plant cell provides for resistance to bacterial pathogens carrying the *avrRpt2* avirulence gene. For example, transgenic plants of the instant invention expressing *RPS2* might be used to alter, simply and inexpensively, the disease resistance of plants normally susceptible to plant pathogens carrying the avirulence gene, *avrRpt2*.

The invention also provides for broad-spectrum pathogen resistance by mimicking the natural mechanism of host resistance. First, the *RPS2* transgene is expressed in plant cells at a sufficiently high level to initiate the plant defense response constitutively in the absence of signals from the pathogen. The level of expression associated with plant defense response initiation is determined by measuring the levels of defense response gene expression as described in Dong et al., supra. Second, the *RPS2* transgene is expressed by a controllable promoter such as a tissue-specific promoter, cell-type specific promoter or by a promoter that is induced by an external signal or agent thus limiting the temporal and tissue expression of a defense response. Finally, the *RPS2* gene product is co-expressed with the *avrRpt2* gene product. The *RPS2* gene is expressed by its natural promoter, by a constitutively expressed promoter such as the CaMV 35S promoter, by a tissue-specific or cell-type specific promoter, or by a promoter that is activated by an external signal or agent.

Co-expression of *RPS2* and *avrRpt2* will mimic the production of gene products associated with the initiation of the plant defense response and provide resistance to pathogens in the absence of specific resistance gene-avirulence gene corresponding pairs in the host plant and pathogen.

The invention also provides for expression in plant cells of a nucleic acid having the sequence of Fig. 2 or the expression of a degenerate variant thereof encoding the amino acid sequence of open reading frame "a" of Fig. 2.

The invention further provides for the isolation of nucleic acid sequences having about 50% or greater sequence identity to *RPS2* by using the *RPS2* sequence of Fig. 2 or a portion thereof greater than 9 nucleic acids in length, and preferably greater than about 18 nucleic acids in length as a probe. Appropriate reduced hybridization stringency conditions are utilized to isolate DNA sequences having about 50% or greater sequence identity to the *RPS2* sequence of Fig. 2.

Also provided by the invention are short conserved regions characteristic of *RPS* disease resistance genes. These conserved regions provide oligonucleotide sequences useful for the production of hybridization probes and PCR primers for the isolation of other plant disease-resistance genes.

Both the RPS2 gene and related RPS family genes provide disease resistance to plants, especially crop plants, most especially important crop plants such as tomato, pepper, maize, wheat, rice and legumes such as soybean and bean, or any plant which is susceptible to pathogens carrying an avirulence gene, e.g., the *avrRpt2* avirulence gene. Such pathogens include, but are not limited to, *Pseudomonas syringae* strains.

The invention also includes any biologically active fragment or analog of an Rps2 polypeptide. By "biologically active" is meant possessing any in vivo activity which is characteristic of the Rps2 polypeptide shown in Fig. 2. A useful Rps2 fragment or Rps2 analog is one which exhibits a biological activity in any biological assay for disease resistance gene product activity, for example, those assays described by Dong et al. (1991), supra; Yu et al. (1993) supra; Kunkel et al. (1993) supra; and Whalen et al. (1991). In particular, a biologically active Rps2 polypeptide fragment or analog is capable of providing substantial resistance to plant pathogens carrying the *avrRpt2* avirulence gene. By substantial resistance is meant at least partial reduction in susceptibility to plant pathogens carrying the *avrRpt2* gene.

Preferred analogs include Rps2 polypeptides (or biologically active fragments thereof) whose sequences differ from the wild-type sequence only by conservative amino acid substitutions, for example, substitution of one amino acid for another with similar characteristics (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the polypeptide's biological activity.

Analogs can differ from naturally occurring Rps2 polypeptide in amino acid sequence or can be modified in ways that do not involve sequence, or both. Analogs of the invention will generally exhibit at least 70%, preferably 80%, more preferably 90%, and most preferably 95% or even 99%, homology with a segment of 20 amino acid residues, preferably 40 amino acid residues, or more preferably the entire sequence of a naturally occurring Rps2 polypeptide sequence.

Alterations in primary sequence include genetic variants, both natural and induced. Also included are analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids. Also included in the invention are Rps2 polypeptides modified by *in vivo* chemical derivatization of polypeptides, including acetylation, methylation, phosphorylation, carboxylation, or glycosylation.

In addition to substantially full-length polypeptides, the invention also includes biologically active fragments of the polypeptides. As used herein, the term "fragment", as applied to a polypeptide, will ordinarily be at least 20 residues, more typically at least 40 residues, and preferably at least 60 residues in length. Fragments of Rps2 polypeptide can be generated by methods known to those skilled in the art. The ability of a candidate fragment to exhibit a biological activity of Rps2 can be assessed by those methods described herein. Also included in the invention are Rps2 polypeptides containing residues that are not required for biological activity of the peptide, e.g., those added by alternative mRNA splicing or alternative protein processing events.

Other embodiments are within the following claims.

What is claimed is: